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Vasoactive Intestinal Polypeptide and Mast Cells Regulate Increased Passage of Colonic Bacteria in Patients With Irritable Bowel Syndrome

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CLINICAL AT

BACKGROUND & AIMS: Irritable bowel syndrome (IBS) is associated with intestinal dysbiosis and symptoms of IBS develop following gastroenteritis. We aimed to study the passage of live bacteria through the colonic epithelium, and determine the role of mast cells (MCs) and vasoactive intestinal polypeptide (VIP) in barrier regulation in IBS and healthy individuals. **METHODS:** Colon biopsies from 32 women with IBS and 15 age-matched healthy women (controls) were mounted in Ussing chambers; we measured numbers of fluorescently labeled *Escherichia coli* HS and *Salmonella typhimurium* that passed through from the mucosal side to the serosal side of the tissue. Some biopsies were exposed to agents that block the VIP receptors (VPAC1 and VPAC2) or MCs. Levels of VIP and tryptase were measured in plasma and biopsy lysates. Number of MCs and MCs that express VIP or VIP receptors were quantified by immunofluorescence. Biopsies from an additional 5 patients with IBS and 4 controls were mounted in chambers and Salmonella were added; we studied passage routes through the epithelium by transmission electron microscopy and expression of tight junctions by confocal microscopy. **RESULTS:** In colon biopsies from patients with IBS, larger numbers of *E coli* HS and *S typhimurium* passed through the epithelium than in biopsies from controls ($P < .0005$). In transmission electron microscopy analyses, bacteria were found to cross the epithelium via only the transcellular route. Bacterial passage was reduced in biopsies from patients with IBS and controls after addition of antibodies against VPACs or ketotifen, which inhibits MCs. Plasma samples from patients with IBS had higher levels of VIP than plasma samples from controls. Biopsies from patients with IBS had higher levels of tryptase, larger numbers of MCs, and a higher percentage of MCs that express VPAC1 than biopsies from controls. In biopsies from patients with IBS, addition of Salmonella significantly reduced levels of occludin; subsequent addition of ketotifen significantly reversed this effect. **CONCLUSIONS:** We found that colonic epithelium tissues from patients with IBS have increased translocation of commensal and pathogenic live bacteria compared with controls. The mechanisms of increased translocation include MCs and VIP.

Keywords: Intestinal Permeability; Bacteria; Ketotifen; Inflammation.

Irritable bowel syndrome (IBS) is characterized by chronically recurring abdominal pain and disturbed bowel habits and affects 10%–15% of the general population in industrialized countries, with a 2:1 female predominance.[1] Even though the pathophysiology is not completely understood, increasing evidence supports the concept of altered brain-gut microbiome interactions in IBS etiology.[2,3]

For many years it has been known that IBS-like symptoms develop following an acute gastroenteritis in about 10% of affected individuals.[4,5] Specifically, it has been confirmed in several studies that enteric infection with *Salmonella*, such as the foodborne pathogenic gram-negative *Salmonella typhimurium,* is a significant risk factor for the development of IBS.[6,7] Evidence also suggests that IBS can be associated with an altered gut microbiota composition or dysbiosis,[8] including the presence of *Escherichia coli.*[9,10] Some of the observed changes in species richness or community composition may reflect alterations in gastrointestinal transit.[11] There is increasing evidence demonstrating enhanced intestinal permeability and altered tight junction patterns[12–14] both in the colon and small bowel of IBS.[15–19]

In intestinal barrier function studies, IBS patients typically have been divided into subgroups based on predominant bowel habit (IBS-diarrhea [IBS-D], IBS-constipation [IBS-C], IBS-mixed [IBS-M]),[20] but the association between bowel habit subtype and mucosal barrier function remains unclear.[15,21] There is some evidence that visceral hypersensitivity, a common feature in IBS, may be linked to disturbances in barrier function,[22] and such changes have been observed in an animal model of gastrointestinal infection.[23]

Abbreviations used in this paper: **^{51}Cr-EDTA, ^{51}chromium (Cr)-EDTA; *Escherichia coli*, *E coli*; HCs, healthy controls; IBS, irritable bowel syndrome; IBS-C, IBS with constipation; IBS-D, IBS with diarrhea; IBS-M, IBS with mixed stool consistency; I_{SC}, short-circuit current; MCs, mast cells; *Salmonella typhimurium*, *S typhimurium*; SEM, scanning electron microscopy; TER, transepithelial resistance; TEM, transmission electron microscopy; VIP, vasoactive intestinal polypeptide; VPAC1, VIP receptor type 1; VPAC2, VIP receptor type 2.**

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EDITOR'S NOTES

BACKGROUND AND CONTEXT

Irritable bowel syndrome (IBS) is associated with intestinal dysbiosis and IBS symptoms can develop following gastroenteritis. The role of intestinal barrier function in the pathophysiology of IBS remains unclear.

NEW FINDINGS

Colonic biopsies from patients with IBS have increased translocation of commensal and pathogenic live bacteria compared with healthy controls. This barrier dysfunction appears to involve mast cells and vasoactive intestinal polypeptide.

LIMITATIONS

Since the IBS patients in the present study had mainly severe symptoms, results are inconclusive for patients with milder disease.

IMPACT

The findings of an enhanced translocation of live bacteria and mucosal immune activation in colonic biopsies provide evidence for a dysfunctional intestinal barrier in IBS.

The gut barrier function is controlled by several physiological mechanisms. Vasoactive intestinal polypeptide (VIP) is a neuropeptide found in both immune cells, including lymphocytes[24] and mast cells (MCs),[25] and in enteric neurons of the gastrointestinal tract. These neurons innervate the intestinal epithelium and regulate ion and fluid secretion, as well as epithelial homeostasis.[26,27] A few studies have also implicated VIP in the regulation of intestinal permeability.[28–30] Our group previously showed that MCs and VIP regulate the ileal barrier of healthy humans and during stress in rats via VIP-receptors (VPAC1/VPAC2) on MCs.[25] Increased plasma levels of VIP have been reported in IBS patients[31] and in an IBS-animal model,[32] and there appears to be a positive correlation between an increased intestinal transcellular permeability and amount of mucosal MCs in IBS-D.[33] In addition, increased MC numbers and tryptase levels have been found in biopsies of caecum[34] and jejunum[35] of IBS patients, and increased paracellular permeability and higher MC numbers were demonstrated in colon of IBS-D patients.[18] To our knowledge there are no reports exploring the intestinal epithelial response to live bacteria and its regulation by VIP and MCs in the colonic mucosa of IBS subjects.

The aims of the study were to investigate (1) the passage of live commensal and pathogenic microbes through the colonic mucosa of women with IBS; (2) the role of MCs and VIP in the regulation of bacterial passage; and (3) the distribution of MCs, VIP/VPACs in the colon of IBS subjects. We present evidence of increased bacterial passage through the colonic mucosa in IBS, with MCs and VIP involved as modulating factors. Our findings indicate a potential link between altered intestinal barrier function and the increased transepithelial passage of bacteria in IBS, and suggest an involvement of VIP and MCs in this mechanism.

Material and Methods

Subjects and Endoscopy

Subjects. Thirty-seven women with IBS, mean age 32.2 years (range, 19–55 years), meeting Rome III criteria were recruited from the Gastroenterology Department, University Hospital, Linköping, Sweden. IBS patients had a moderate-severe IBS with mean symptom severity score of 347 (range, 167–480).[36] Twenty healthy age-matched women, mean age 29.9 years (range, 20–48), without medical history of gastrointestinal symptoms or complaints, were recruited as healthy controls (HCs). IBS subjects were classified according to predominant bowel habit into IBS-M (n=21), IBS-C (n=8), and IBS-D (n=8). Exclusion criteria for both groups included self-reported allergy, organic gastrointestinal disease, metabolic or neurologic disorders, and nicotine or NSAID intake. The committee of human ethics, Linköping, approved the study and all subjects gave their written informed consent.

IBS subgroups. IBS patients were subgrouped based on the predominant stool consistency according to the Rome III questionnaire.[20]

Sigmoidoscopy. A flexible sigmoidoscopy was performed after 8 hours of fasting without sedation and with scope insertion approximately 30–40 cm orally from linea dentata. Colonic biopsies were taken with a biopsy forceps without a central lance and placed directly into ice-cold oxygenated Krebs buffer.[37] Prior to sigmoidoscopy, venous blood samples were collected for measurements of VIP concentrations.

Ussing Chamber Experiments

Barrier function studies. Colonic biopsies from 32 IBS patients and 15 HCs were mounted in Ussing chambers[38] as previously described.[37] After 20 minutes biopsies were treated with 1 μmol/L anti-VPAC, 1 μmol/L of the MC-blocker ketotifen, or vehicle (Krebs). After another 20 minutes, 34 μCi/mL of the paracellular probe ^{51}chromium (Cr) was added to the mucosal side of each chamber. Live green fluorescent protein-labelled *E coli* HS or *S typhimurium*, prepared as previously described,[37] was added to the mucosal sides of separate chambers at a final concentration of 10^8 CFU/mL. Serosal samples were collected and bacterial and ^{51}Cr-EDTA passage was measured. The transepithelial potential difference, short-circuit current (I_{sc}), and the transepithelial resistance (TER) across the tissues were monitored throughout the experiments to ensure tissue viability.

Electron microscopy. To identify bacterial translocation and MC ultrastructure, colonic biopsies from 5 IBS patients and 4 HCs were mounted in Ussing chambers and exposed to bacteria. Anti-VPACs or ketotifen was added as described earlier, followed by addition of green fluorescent protein–labelled *S typhimurium*. Krebs was added to 1 biopsy as control to evaluate the eventual effects on tissue structure during incubation. After 30 minutes, biopsies were fixed and processed following standard protocols for scanning and transmission electron microscopy (SEM, TEM). MC-degranulation was also quantified in basal biopsies, not mounted in Ussing chambers, from 20 IBS patients and 8 HCs.

Quantification of tight junctions. To study the effects of *S typhimurium*, anti-VPACs and ketotifen on tight junctions, biopsies from 4 IBS patients and 4 HCs were mounted in Ussing chambers and added anti-VPACs, ketotifen and *S typhimurium*. After 30 minutes, biopsies were fixed in the chambers and prepared for immunofluorescence of tight junctions (see below).

Giemsa Staining of Biopsies

To identify the presence of intestinal bacteria at baseline, 1 paraffin-embedded biopsy/individual, was stained with routine Giemsa according to a previous protocol.[39] Bacteria in 10 areas of 0.033 mm^2/biopsy were counted and results are presented as bacteria/mm^2.

Measurements in Biopsies and Plasma

Preparation of biopsy lysates. Lysates from frozen biopsies were prepared to a final concentration of 1 mg protein/mL, according to the procedure described in the Supplementary Methods.

Quantification of VIP in plasma and biopsies by EIA. To examine VIP levels in plasma and biopsies from IBS patients and HCs, a VIP-EIA kit was used following manufacturer's instructions (Phoenix Pharmaceuticals, Germany).

Quantification of MC-tryptase in biopsies by Western Blot. Biopsy lysates with protein concentrations of 20 μg/mL were loaded on gels and further processed as described in the Supplementary Methods. Values were normalized according to the β-actin loading control and values are given as fluorescence units.

Microscopy

Quantification of MC-tryptase, VIP, and VPACs. Biopsies from 32 IBS patients and 15 HCs were stained following the protocol described earlier for tight junctions. Primary antibodies used were MC tryptase+ VPAC1/VPAC2 or VIP. The total number of MCs and co-localization with either VPACs or VIP were quantified manually and results presented as the percentages of VIP/VPAC1/VPAC2-positive MCs from patients with IBS and HCs, respectively.

Evaluation of mucosal MC ultrastructure. MCs were identified based on morphologic characteristics in a minimum of 20 ultrathin sections from each sample. Ultrastructural changes indicative of activation were described and a quantitative method was used to assess MC activation based on the degranulation area. For each MC, the cytoplasm and degranulated area (loss of granular content) were measured using the ImageJ Fiji software (National Institutes of Health, Bethesda, MD). Degranulation was expressed as the percentage of degranulated area with respect to the total area of the cytoplasm.

Evaluation of bacterial passage routes. After incubation, the presence of bacteria on the epithelial surface was observed by SEM and TEM. Interaction of *S typhimurium* with epithelial cells and bacteria crossing through the colonocytes were identified by TEM.

Immunofluorescence of tight junctions. Biopsies were stained for occludin and ZO-1, evaluated by confocal microscopy, and images were further processed in ImageJ Fiji software. Results were normalized and presented as percent of the expression in vehicle biopsies set to 100%.

Statistic Analysis

Parametric data are given as mean ± SEM and comparisons between groups were performed with Student *t* test and ANOVA test. Non-parametric data are given as median (25th–75th interquartile range) and comparisons between groups were done with Kruskal–Wallis and Mann–Whitney U tests. Wilcoxon matched-pairs signed rank test was used for paired comparisons. Differences with $P < .05$ were considered significant. Correlation testing was conducted with Spearman Correlation test.

Results

Mucosal Barrier Function in IBS and HCs

Electrophysiology. Ussing chamber experiments showed stable transepithelial potential difference after equilibration in all biopsies. The active net ion transport, assessed as I_{sc}, was similar in IBS and HCs throughout the experiments. There were no effects on I_{sc} of *E coli* HS, *S typhimurium*, anti-VPACs, or ketotifen (data not shown).

In vehicle chambers there was a lower TER in IBS compared with HCs when experiments were started (22 ohm/cm^2 [19–27] vs 25 [21–32], $P < .05$), at 30 minutes (21 [18–25] vs 24 [22–29], $P < .05$), and at 60 minutes (20 [17–24] vs 24 [20–26], $P < .05$). At 90 minutes from start, TER remained lower in IBS (20 [17–25]) compared with HCs (24 [19–26]); however, this difference no longer reached statistical significance ($P = .07$). In IBS biopsies added *S typhimurium* there was a significantly decreased TER at 30, 60, and 90 minutes compared with vehicle ($P < .05$; Table 1). In chambers pre-incubated with ketotifen, the *Salmonella*-induced decrease in TER was inhibited ($P < .05$), while anti-VPACs had no significant effect. In HC,

Table 1. Effects of Live *Salmonella typhimurium* on Transepithelial Resistance Over Time

Time after start	Vehicle	*S typhimurium (% decrease from start)*	*S typhimurium*+ Anti-VPACs	*S typhimurium*+ ketotifen
30 minutes	**3.4** (1.0–6.6)	**12.4** (1.3–25.7)[a]	**8.1** (1.5–14.2)	**4.4** (0.0–11.8)[b]
60 minutes	**5.1** (3.1–11.1)	**19.6** (4.8–33.6)[a]	**10.8** (0.0–17.8)	**7.8** (0.0–16.4)[b]
90 minutes	**6.7** (3.5–10.4)	**15.1** (3.1–30.0)[a]	**10.0** (0.3–21.6)	**8.8** (2.8–19.9)

NOTE. Colonic biopsies from 32 patients with IBS were mounted in Ussing chambers and added buffer (vehicle), *S typhimurium*, *S typhimurium*+vasoactive intestinal polypeptide receptor blocker (anti-VPACs), or *S typhimurium*+ketotifen. Transepithelial resistance (TER) was monitored at 30, 60 and 90 minutes from start and values are given as percent decrease in TER from start.

[a]$P < .05$ vs vehicle at 30, 60, and 90 minutes, respectively.

[b]$P < .05$ vs *S typhimurium* at 30 and 60 minutes, respectively. Comparisons were done with Mann–Whitney U test and values given as (median [25th–75th percentile]).

S typhimurium had no significant effect on TER at 30, 60, or 90 minutes (data not shown). Biopsies mounted in chambers with added *E coli* HS showed the same pattern for TER as vehicle chambers in both IBS and HCs (data not shown).

Increased bacterial and paracellular passage in IBS. The transepithelial passage in biopsies of IBS subjects was nearly 2 times higher for *E. coli*, and 2.8 times higher for *S typhimurium* compared with HCs ($P < .0005$), and fluorescence microscopy confirmed uptake of both *E coli* and *S typhimurium* in Ussing chambers (Figure 1 *A-B*). The passage of the 2 bacteria strains was not significantly correlated.

Permeability to ^{51}Cr-EDTA was higher in IBS compared with HCs (1.10 cm/s $\times$ 10^{-6} (0.74–1.50) vs 0.80 (0.53–1.10; $P < .05$). Addition of *E coli* or *S typhimurium* had no significant effect on ^{51}Cr-EDTA permeability. No significant correlations were identified between ^{51}Cr-EDTA, *S typhimurium*, and *E coli* passage.

Bacterial passage route. *E coli* and *S typhimurium* were detected on the epithelial surface and in the lamina propia after incubation (Figure 1*B*). *S typhimurium* was observed to interact with the microvilli of colonocytes and translocation was identified by endocytosis through the transcellular pathway (Figure 1*C*). No bacteria were observed to interact at the tight junctions in any sample. No differences were identified between groups and treatments, in terms of bacteria interaction with the epithelial surface and internalization to the colonocytes.

Intestinal bacteria identified by Giemsa. Giemsa staining of biopsies that had not been experimentally exposed to bacteria revealed that bacteria within the epithelium were only scarcely seen in both IBS and HCs (Figure 1*D*).

Regulation of barrier function by VIP and MCs. Bacterial passage was significantly diminished in both IBS subjects and HCs after blocking with anti-VPACs and ketotifen (Figure 2*A-B*).

Tight junction proteins. Confocal microscopy showed close to significantly decreased baseline levels of occludin in IBS (346.7 fluorescence units [308.2–371.9]) compared with HCs (420.8 [368.8–609.7]); $P = .057$), but no difference in ZO-1 expressions (IBS: 530.4 [345.5–599.9]; HCs: 398.7 [280.9–463.4]). The addition of *S typhimurium* to the biopsies decreased occludin expression in the apical side of the colonocytes compared with vehicle (set to 100%) in both IBS (42.9 [21.8–80.1]) and HCs (47.3 [10.7–105.8]), but it did not reach statistical significance. Notably, in IBS biopsies pre-incubated with ketotifen, the *S typhimurium*-induced decrease in occludin expression was significantly inhibited (130.5 [100.2–154.6]; $P < .005$), while anti-VPACs had no significant effect (61.6 [29.0–88.4]). There was no effect on expression or distribution of ZO-1 by *S typhimurium*, ketotifen, or anti-VPACs. For more detailed results, see Supplementary Table 1. Representative photographs of occludin staining during the different conditions are shown in Figure 3 and for ZO-1 in Supplementary Figure 1.

Increased VIP in Plasma of IBS Patients

VIP in plasma of IBS patients was higher than in HCs (2.1 $\pm$ 0.09 ng/mL vs 1.5 $\pm$ 0.09; $P < .0005$). However, there were no significant differences in biopsy lysates (0.57 $\pm$ 0.10 ng/mg protein vs 0.32 $\pm$ 0.05). There was no significant correlation between VIP levels in plasma and in biopsies. No significant correlation was observed between *E coli*/*S typhimurium* and VIP levels and between ^{51}Cr-EDTA and VIP levels.

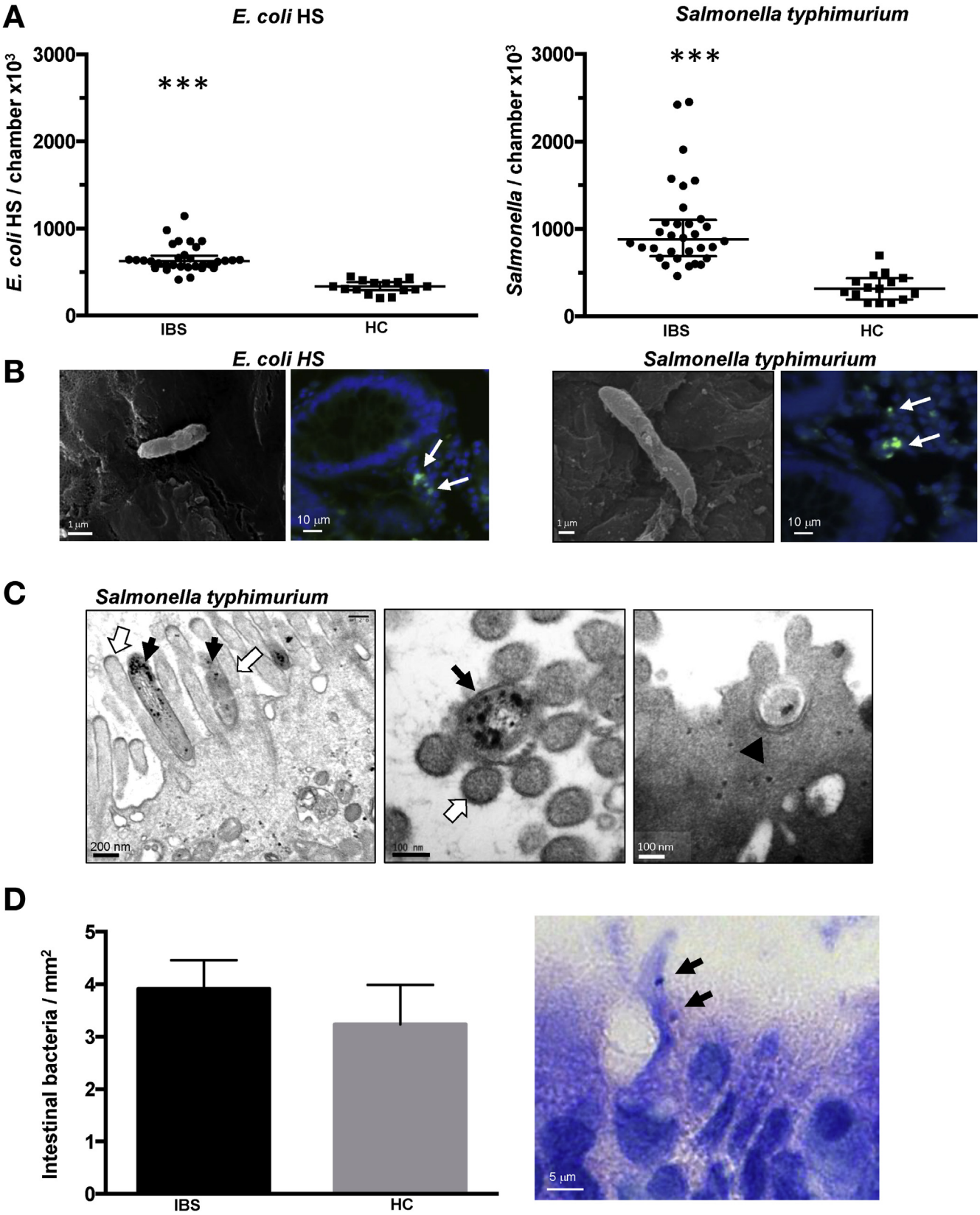
Increased Number of MCs, MCs Expressing VPAC1, and MC-Activation in IBS

Colonic IBS biopsies showed increased number of MCs compared with HCs (104.0 $\pm$ 8.0 MCs/mm^2 vs 73.7 $\pm$ 11.6; $P < .05$). Furthermore, the percentage of MCs expressing VPAC1 was higher in IBS compared with HCs (46 $\pm$ 4.2% vs 31 $\pm$ 6.2; $P < .05$). However, there was no difference in the percentage of MCs expressing VPAC2 (30 $\pm$ 4.4% vs 26 $\pm$ 6.1). When comparing microscopy results patient by patient it was revealed that only 75% of the IBS patients had elevated MC numbers compared with the mean of HCs, and 78% showed higher percentage of MCs expressing VPAC1. Representative photographs of MCs expressing VPAC1 in an IBS patient and HC are shown in Figure 4*A-B*. MCs expressing VPAC2 in IBS colon is illustrated in Figure 4*C*. There was a trend to higher amounts of MCs containing VIP in IBS compared with HCs; however, it did not reach statistical significance (24 $\pm$ 1.0% vs 20 $\pm$ 1.3; $P = .06$). A VIP-containing MC of an IBS patient is shown in Figure 5. In all subjects, MC density correlated with the percentage of MCs expressing VPAC1 (R=0.35, $P = .01$). In addition, the percentage of MCs expressing VPAC1 strongly correlated with the percentage of MCs expressing VPAC2 (R=0.67, $P < .0001$).

Analysis of biopsy lysates by Western blot revealed higher levels of MC-tryptase in IBS compared with HCs (2.26 $\pm$ 0.46 fluorescence units vs 1.42 $\pm$ 0.46; $P < .05$) (Figure 6*A*).

In TEM, mucosal MCs showed signs of activation in samples from all subjects, with variable granular electron density and piecemeal degranulation. However, in MCs from IBS patients, distribution of the cytoplasmic granules to the periphery, close to the plasma membrane, was identified, together with higher frequency of membrane projections to the extracellular matrix, which is indicative of active release of granular content. Anaphylactic degranulation was not observed in any sample, although some MCs in the IBS group also displayed fusion of several granules. MCs from IBS showed higher degree of degranulation; however, this difference did not reach statistical significance ($P = .113$) (Figure 6*B*). Exposure to vehicle did not affect MC ultrastructure, as they were similar to MCs in non-incubated tissues. *S typhimurium* increased MC degranulation, and pre-incubation with anti-VPACs or ketotifen maintained a more stable granular structure in IBS and HCs. However, degranulation was not statistically significant between experimental conditions (Figure 6*C-D*).

There was no correlation between MC-tryptase in biopsy lysates and bacterial passage (*E coli* and *S typhimurium*). However, MC density correlated with MC-tryptase expression in biopsy lysates in all subjects (R=0.34, $P = .03$).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IBS
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Relation Between Gut Findings and Clinical Data

There was no correlation between age of subjects with IBS/HC and any of the parameters investigated (results not shown).

There was no significant correlation between symptoms (IBS-severity scoring system) and bacterial passage, MC numbers, or MC-tryptase. There was a negative correlation between ^{51}Cr-EDTA (R=-0.41, $P < .05$) and MC degranulation (R=-0.59, $P < .01$) with the IBS-severity scoring system. Significant differences were found between the subgroups IBS-M and IBS-C in terms of MCs. MC density was higher in IBS-M compared with IBS-C (127 ± 48 MCs/mm^2 vs 75 ± 23; $P < .005$) and the proportion of MCs expressing VPAC1 and VPAC2 was significantly higher in IBS-M compared with IBS-C (VPAC1: 55.7 ± 4.3% vs 21.5 ± 6.3; $P < .005$; VPAC2: 38.4 ± 4.9% vs 8.4 ± 3.2; $P < .0005$). For IBS-D the numbers were 46.3 ± 10.3% for VPAC1 and 32.1 ± 12.9% for VPAC2.

Discussion

In the present study we demonstrated that female IBS subjects show a greater colonic epithelial permeability to live bacteria and that this barrier dysfunction may be associated with an up-regulation of MC- and VIP-related signaling mechanisms. IBS patients had an increased passage of both live *E coli* HS, a commensal bacteria that normally colonize the colon,[40] and of the pathogenic bacteria *S typhimurium*. In IBS and HCs, bacterial passage of both strains was diminished by blocking the VIP receptors, as well as after stabilizing MCs with ketotifen. Ketotifen is a histamin H1-receptor antagonist and it has been reported that thresholds for discomfort were increased in IBS when taking this drug compared with placebo.[41] We also found that the colonic mucosa of IBS patients had an enhanced number of MCs and that MCs of IBS patients expressed more VPAC1 than HCs. Further, biopsy lysates from IBS patients demonstrated elevated tryptase levels and the MCs displayed a more activated morphologic/ultrastructural phenotype when compared with HCs. Even though disturbances of the epithelial barrier function have previously been described in IBS,[19,42] to our knowledge, this is the first study demonstrating an increased transepithelial passage of live bacteria in human subjects with IBS, and identifies VIP as a key regulatory molecule of this mechanism.

The role of immune activation and its involvement in IBS clinical symptoms remains unclear. Evidence for mucosal immune activation in IBS has been reported in some, but not all studies,[3,43] Increased MC numbers, T-cell activation, and cytokine polymorphism have also been reported in IBS colon by numerous groups.[44–46] For example, Cremon et al[47] found increased MC and T-cell numbers in the colon of 50% of IBS subjects. In the present study, we found increased MC numbers in 75% of IBS subjects; however, we could not find any correlation between MC infiltration and symptoms.

There is growing evidence for altered microbiota composition in some IBS patients.[48] The gut microbiota is involved in several aspects of gastrointestinal function, including motility, sensitivity, and immune function.[2] Recent data also suggest that the human gastrointestinal microbiota is able to communicate with the central nervous system.[49] *E coli* K12 and HB101 have been used as commensal-like bacteria to study luminal uptake/passage into the intestinal mucosa.[37,39] In the present study we chose the human commensal *E coli* HS as a non-pathogenic *E coli*, which has similar properties as K12 and HB101.[50] Live *E coli* HS passed through the colonic mucosa of IBS patients to a significantly higher extent compared with HCs. These results suggest that commensal bacteria and its metabolites can potentially affect and interact with intra- and submucosal targets in IBS to a greater degree than in HCs.

There are multiple pathways how bacteria and bacterial products can interact with immune- neuroendocrine or neural constituents.[5,8,49,51] However, it is important to remember that gut permeability is determined not only by the transepithelial component, but also by the inner compartment of the mucus layer.[52] Ex vivo, this mucus layer is easily removed from the epithelial cells because of flow in the Ussing chambers,[53] which is important to take under consideration when interpreting the results and translating it to the clinical situation. Because there was very little overlap between IBS and HCs in terms of transepithelial bacterial passage, and evidence for immune activation has not consistently been found in colon of IBS subjects, despite the fact that all IBS patients had moderate to severe symptoms, it is conceivable that in the intact colon, live bacteria such as *E coli* gain access to the underlying submucosa only in those patients who also have a compromised mucus layer. This could also explain why we found IBS symptom severity to be inversely correlated with paracellular permeability, and why there was no significant correlation between symptom severity and bacterial passage and MCs, limited though by a small number of subjects when divided into IBS subgroups. These findings strongly suggest that alterations in transepithelial permeability alone cannot explain the formation of symptoms in IBS.

Monitoring of electrophysiology showed no difference in I_{sc} between IBS and HCs. Previous studies on I_{sc} and IBS are mostly lacking. However, Peters et al[54] recently showed a similar I_{sc} in IBS-C and HCs, which is in line with our findings and indicates an increased bacterial uptake in IBS, even in circumstances where ion transport is unchanged. To further determine changes in permeability caused by tight

Figure 1. Bacterial passage through colonic biopsies from 32 IBS patients and 15 HC mounted in Ussing chambers for 120 minutes. (*A*) Passage of live *E coli* HS and *S typhimurium.* Comparisons were performed with Mann–Whitney U test, ***$P < .0005$ vs HC. (*B*). Representative SEM of *E coli* and *S typhimurium* on the surface of the colonic epithelium and representative confocal micrographs illustrating bacterial uptake (*white arrows*) into the mucosa of IBS colon after incubation. (*C*) TEM illustrating interaction of *S typhimurium* (*black arrows*) and microvilli (*white arrows*); a transversal section where interaction is shown; and bacterial uptake through endocytosis (note double membrane) at the apical side of the epithelium. (*D*) Intestinal bacteria quantified by Giemsa staining. *Arrows* indicate 2 bacteria just entering the epithelium in an IBS sample.

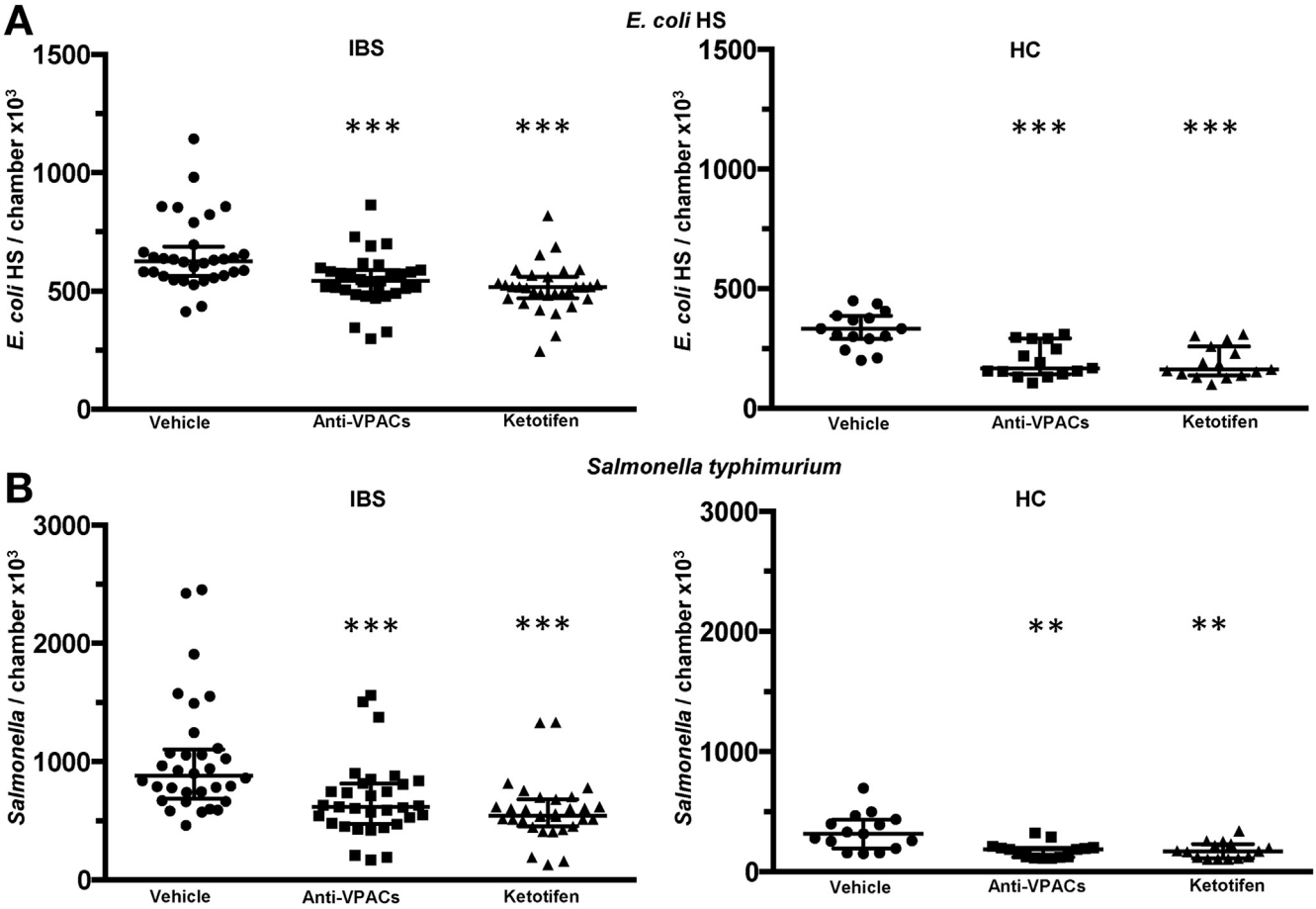

Figure 2. Influence of VIP and MCs on bacterial passage through colonic biopsies from 32 IBS patients and 15 HC mounted in Ussing chambers. Figure indicates passage at 120 minutes of *E coli* HS (*A*) and *S typhimurium* (*B*) before (vehicle) and after addition of VIP receptor blocker (anti-VPACs) or MC-stabilizer (ketotifen) to the chambers. Comparisons were performed with Mann–Whitney U test, $^{**}P < .005$, $^{***}P < .0005$ vs vehicle.

junction components, we chose to study occludin and ZO-1 expression as main constituents of the apical junctions, based on previous reports demonstrating their deregulation in the colon of IBS.[13] Our study confirms previous data and extends its contribution to bacteria-mediated epithelial alterations. Although the role of occludin still remains unknown, our results suggest its regulation by mechanisms that involve MC activation. Because bacteria was not observed entering the epithelium through the paracellular space, we can speculate that occludin deregulation is secondary to epithelial invasion and, probably, immune activation, by eg, TNF-α which has been related with the internalization of occludin, inducing secretion of water, barrier dysfunction, and diarrhea.[55]

The well-described onset of IBS symptoms after gastroenteritis in a number of affected individuals is another

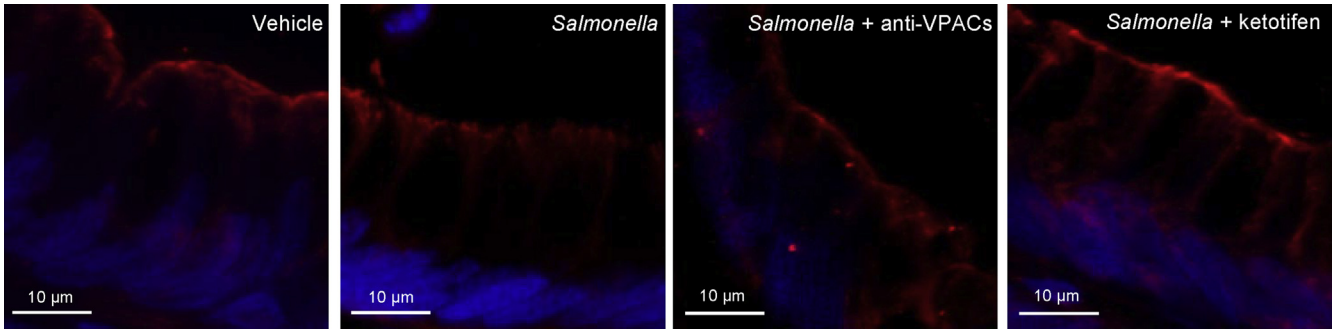

Figure 3. The effect of live *S typhimurium* on occludin. Photographs illustrate staining for occludin in colonic biopsies from a patient with IBS mounted in Ussing chambers. Biopsies were added buffer only (vehicle), *S typhimurium*, *S typhimurium*+ VIP-receptor blocker (anti-VPACs), or *S typhimurium*+ketotifen. After 30 minutes, biopsies were removed and stained for occludin by immunofluorescence.

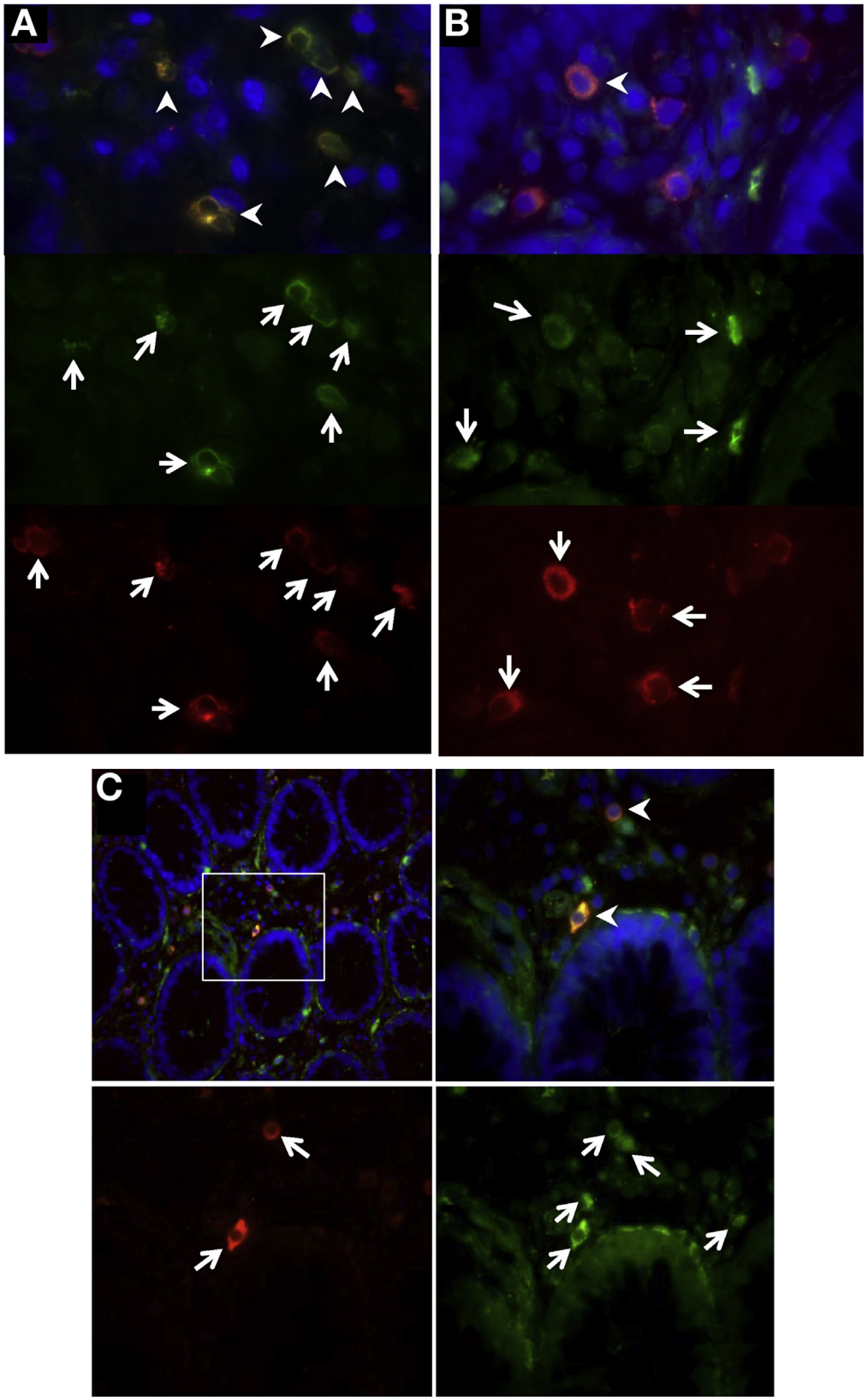

Figure 4. Staining of MC-tryptase (*red*) and vasoactive intestinal polypeptide receptors (VPACs) (*green*) in the colon of a patient with IBS and a HC. Photographs show MCs±VPAC1 expressions in an IBS patient (*A*) and HC (*B*). *Arrows* indicate individual MC or VPAC1 staining and *arrowheads* indicate co-localization (ie, MCs expressing VPAC1). (*C*) Photograph showing MCs expressing VPAC2 (*arrowheads*) in IBS colon. *Arrows* indicate individual MC or VPAC2 staining. Overview photographs: 400× magnification; zoomed photographs, 1000×.

strong indicator of the importance of microbial factors in the pathophysiology of IBS.[48,56,57] It has also been demonstrated that having *Salmonella* infection during childhood was a risk factor for the development of long-standing IBS symptoms in adulthood.[58] *S typhimurium* is a pathogenic gram-negative bacteria that is well known to cause

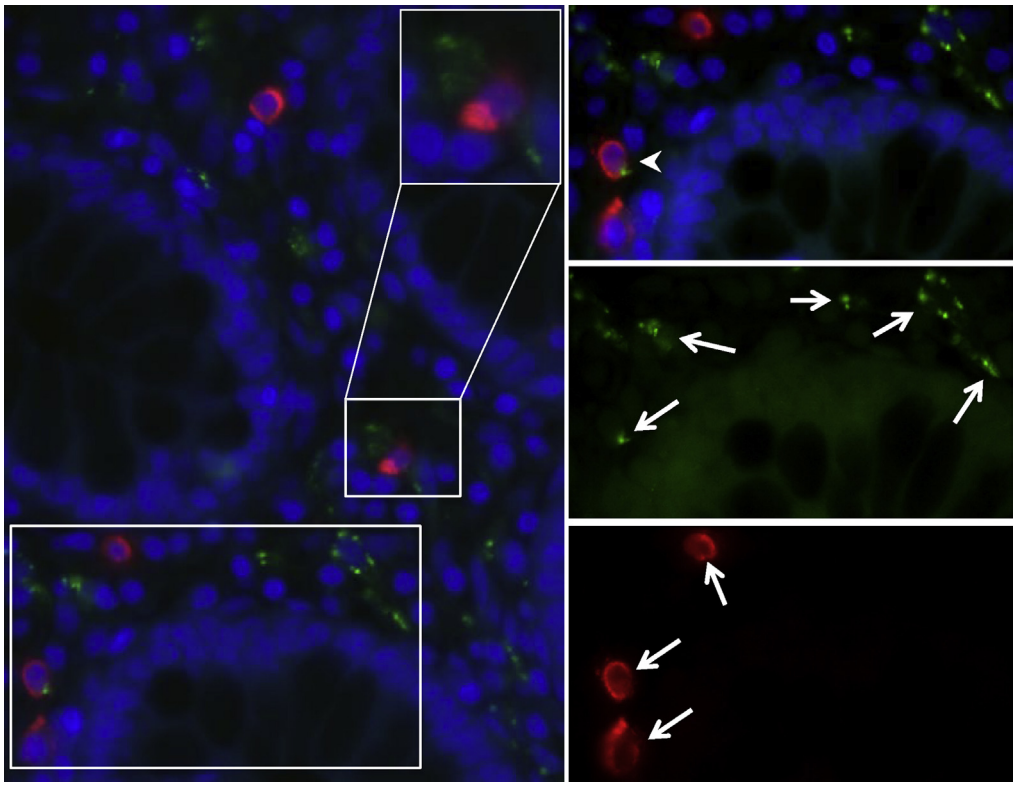

Figure 5. Staining of MC-tryptase (*red*) and VIP (*green*) in colonic biopsy of a patient with IBS. Photograph shows a MC-expressing VIP (*arrowhead*). Window in upper right corner shows a MC in close proximity to secreted VIP that was a common feature seen in IBS patients, but could also be observed in HCs. Overview photographs: 400× magnification; zoomed photographs, 1000×.

gastroenteritis in humans and other mammals.[59] The toxicity is based on an outer membrane with lipopolysaccharides protecting the bacteria from the environment. In the present study we found an almost 3 times higher passage of live *S typhimurium* through IBS mucosa compared with HCs. This indicates that there is a disturbance in IBS patients leading to increased invasion of pathogenic bacteria into the epithelium and the underlying submucosa. One could speculate that the enhanced translocation of *S typhimurium* could lead to increased antigen load and, for example, activation of MCs and other immune cells, which might facilitate local immune activation. Interestingly, the passage of *E coli* and *S typhimurium* did not correlate, indicating different uptake mechanisms and diverse translocation patterns.

Only a small decrease in baseline TER was found in IBS compared with HCs, which may be because the conventional TER measurements are not sensitive enough to fully detect differences between biopsies. Another noteworthy point is that neither *S typhimurium* nor *E coli* significantly affected ^{51}Cr- permeability, even though *S typhimurium* had a significant effect on TER. Several pathogenic *E coli* strains have been shown to affect the paracellular pathway in intestinal epithelia[60]; however, we and others observed no effects on paracellular permeability by non-pathogenic *E coli* strains.[39,61]. For *S typhimurium*, one could have expected an effect on ^{51}Cr-EDTA permeability because previous studies indicate that *S typhimurium* decreases TER and causes tight junction redistribution in vitro[62] Although in the present study there was only a trend to increased ^{51}Cr-EDTA permeability by *S typhimurium,* despite a significant effect on TER, which probably refers to different regulations of permeability pathways (ie, the leak and the pore pathways).[63]

Functional experiments in the present study demonstrated that bacteria passage was significantly diminished after blocking the VPACs and after ketotifen administration, an effect that was not only seen in IBS patients, but also in HCs, suggesting the involvement of VIP and MCs in the physiologic regulation of intestinal barrier function. However, the fact that both VIP levels and MCs expressing VPACs are increased in IBS relative to health further points at a novel role of this mediator in bacterial translocation in IBS. There are only a few reports examining the influence of VIP in IBS. Zhang et al[31] showed increased VIP levels in plasma of IBS patients compared with controls, and in an IBS stress model, Wu et al[32] showed colonic MC activation and increased VIP levels in stressed animals. Interestingly, electro-acupuncture inhibited these events, indicating that the interactions of MCs and VIP could be key mechanisms in IBS pathophysiology.

In addition, we found increased VIP levels in plasma of IBS patients compared with controls. In biopsy lysates there was only a trend to increased VIP levels; however, IBS patients showed almost 2 times higher VIP concentrations compared with controls. Because IBS patients also had elevated VIP receptor density on their MCs in combination with an increased MC density, we presume that the physiologic VIP–MC-dependent regulation of bacterial passage might be up-regulated in IBS.

MCs are innate immune cells involved in protection against pathogens and in allergic responses. When activated, they undergo a degranulation process that leads to the

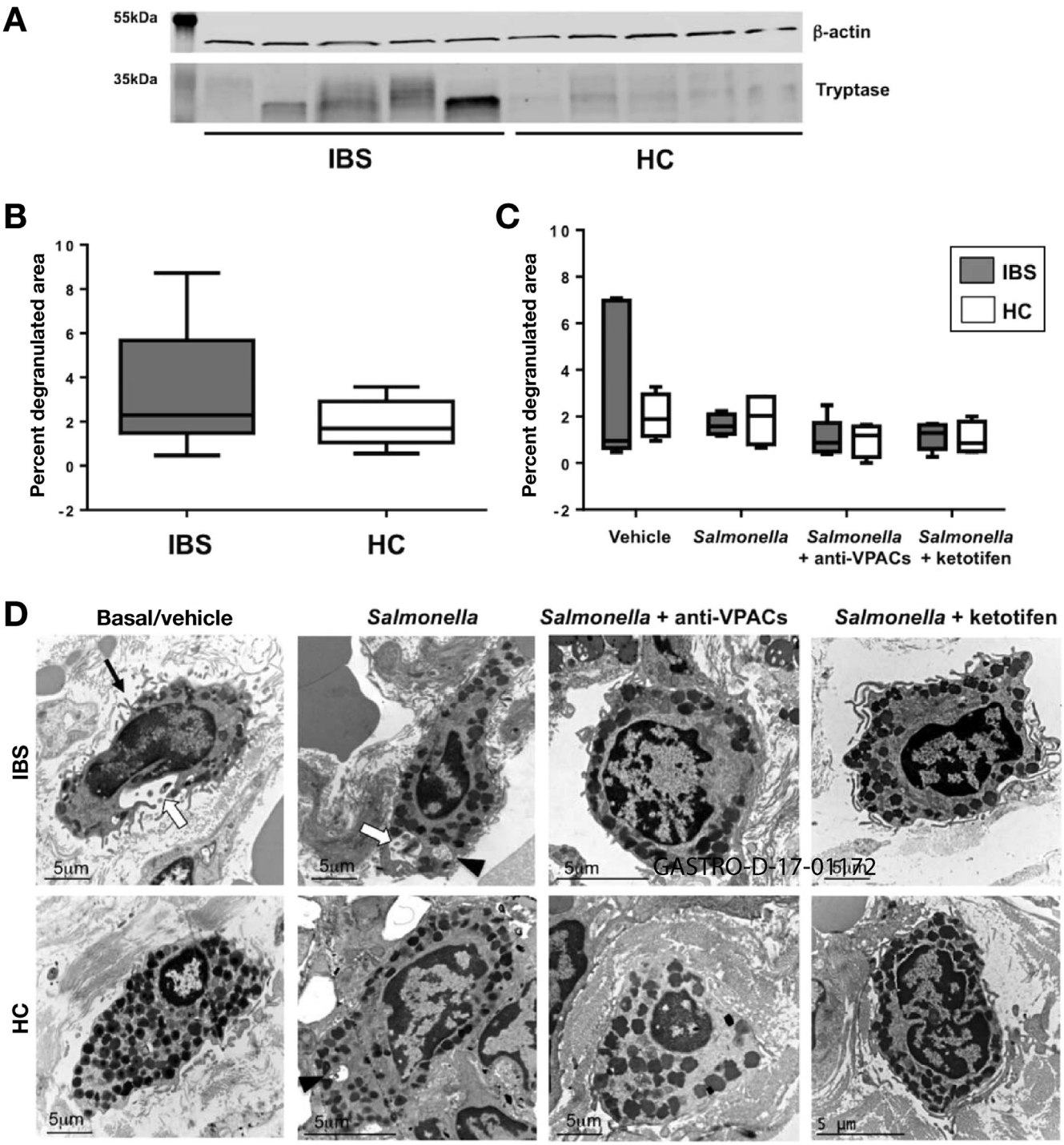

Figure 6. Mucosal MC activation: tryptase, ultrastructure and degranulation in IBS and HC subjects. (*A*) Expression of MC-tryptase (mw 31–36 kDa) and β-actin as loading control. Lysates from colonic biopsies of 32 women with IBS and 15 HCs were analyzed by Western Blot. Panels show a representative blot from 5 IBS subjects and 5 HCs. (*B*) Degranulation of MCs at baseline of IBS (n=19) and HC (n=8). (*C*) Degranulation of MCs in biopsies from IBS patients (n=5) and HCs (n=4) after incubation with buffer only (vehicle), *S typhimurium*, *S typhimurium*+ VIP receptor blocker (anti-VPACs), or *S typhimurium*+ketotifen. (*D*) Micrographs of MCs from IBS and HC from the different experimental groups. Note signs of MC activation such as irregular plasma membrane with emission of pseudopodes (*black arrow*) and piecemeal degranulation (*black arrowhead*). Fusions of granules are predominantly detected in MCs from the IBS-group (*white arrow*).

release of mediators, among which histamine and tryptase have proved to modulate visceral hypersensitivity and epithelial permeability. Our study identified increased MC-tryptase together with more pronounced ultrastructural signs of MC activation from IBS, even though the quantification method used did not corroborate our observations,

which might refer to a large variability within the IBS group at baseline. *S typhimurium* did induce MC degranulation from both IBS and HCs, and the treatments used reduced MC activation; however, a larger cohort should be studied to better prove these effects. Most studies showing differences in MC numbers have included IBS-C or IBS-D. Notably, our study suggests that IBS patients with a mixed bowel habit pattern have the most pronounced alterations in MC density. Moreover, in contrast to the majority of previous reports, subjects with known allergies or atopic diseases were excluded in the current study, reducing the risk of allergy and atopic disease as an important confounding factor when studying MCs in IBS.[64] In fact, a combination of allergic factors such as positive skin tests, elevated IgE or eosinophil levels, and personal or family history of allergy, significantly correlated with MC count and enhanced intestinal permeability.[65] Recently, a positive correlation between increased intestinal permeability and number of mucosal MCs was found in IBS-D patients.[33] However, in the present study we found no significant correlation between MC count and mucosal permeability.

In conclusion, the present study demonstrates an increased transepithelial passage of live commensal and pathogenic bacteria in the colon of IBS subjects. Both MCs and VIP appear to be important factors in the regulation of this bacterial translocation through the mucosa, and theses mechanisms seem to be up-regulated in IBS. Future studies are required to elucidate the relationship between these epithelial abnormalities, mucosal immune activation, and symptom generation in IBS subjects.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2017.06.051.

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Supplementary Methods

Sigmoidoscopy

A flexible sigmoidoscopy was performed by two experienced gastroenterologists (OB, SW) after 8 hours of fasting, and a colon preparation with an enema early in the same morning. The procedure was performed without sedation and with scope insertion approximately 30-40 cm orally from linea dentata. Colonic biopsies were taken with a biopsy forceps without a central lance, and directly put in ice-cold oxygenated Krebs buffer (115 mM NaCl, 1.25 mM CaCl2, 1.2 mM MgCl2, 2 mM KH2PO4, and 25 mM NaHCO3, pH 7.35). Prior to sigmoidoscopy, fasting venous blood samples were collected in EDTA-treated tubes for measurements of VIP concentrations. To each ml of blood, a mixture of 1.3 mg EDTA and 50 μL Trasylol 10 000 KiE was added. Blood was centrifuged at 3400*g* for 15 min in 4°C, and plasma redrawn and frozen in -80°C until analysis.

Barrier Function Studies

Colonic biopsies from 32 IBS patients (32.6 years (19-55), 17 IBS-M, 8 IBS-C, 7 IBS-D) and 15 HCs (29.7 (21-48)) were mounted in Ussing chambers (Harvard apparatus Inc., Holliston, MA, USA). After 20 minutes, biopsies were treated with 1 μM anti-VPAC (Sigma-Aldrich, Sweden), 1 μM of the MC-blocker ketotifen (Sigma-Aldrich) or vehicle (Krebs). After another 20 min, 34 μCi/mL of the paracellular probe ^{51}chromium (Cr)-EDTA (Perkin Elmer, MA, USA) was added to the mucosal side of each chamber. Live green fluorescent protein (GFP)-labelled *E coli* HS or *Salmonella typhimurium* was added to the mucosal sides of separate chambers at a final concentration of 10^8 CFU/ml. Serosal samples were collected at 0, 60 and 120 min and replaced with an equal volume of Krebs. Bacterial passage was determined at 488 nm in VICTOR^{TM} *X3* multileader plate reader (Perkin Elmer, Sweden), and ^{51}Cr-EDTA passage was measured by gamma-counting (1282 Compugamma, Sweden). ^{51}Cr-EDTA permeability was calculated during the 60-120 min period and results are presented as P_{app} (apparent permeability coefficient; cm/s x10^{-6}).

Electron Microscopy

To identify bacterial translocation and MC ultrastructure, colonic biopsies from 5 IBS patients (34 (25-42), 4 IBS-M, 1 IBS-D) and 4 HCs (32.2 [26-44]) were mounted in Ussing chambers, exposed to bacteria, and anti-VPACs or ketotifen was added as described above, followed by addition of GFP-labelled *Salmonella typhimurium*. One biopsy was added Krebs only, as control to evaluate the eventual effects on tissue structure during incubation. After 30 min, biopsies were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 24-72 hours, post-fixed, and dehydrated. Samples were processed following standard protocols for scanning and transmission electron microscopy (SEM, TEM) using an S-570 (Hitachi Ltd, Japan) and a Hitachi H-1400 electron microscope, respectively. MC-degranulation was also quantified in basal biopsies, not mounted in Ussing chambers, from 19 IBS patients (30.9 (19-47), 11 IBS-M, 4 IBS-C and 4 IBS-D) and 8 HCs (27.9 (21-46).

Immunofluorescence and Quantification of Tight Junctions

To study the effects of *Salmonella*, anti-VPACs and ketotifen on tight junctions, biopsies from 4 IBS patients (34.8 (25-42), 3 IBS-M, 1 IBS-D) and 4 HCs (31.2 (26-44)) were mounted in Ussing chambers and added anti-VPACs, ketotifen and *Salmonella* as described above. After 30 min in Ussing chambers, biopsies were fixed in chambers with 4% paraformaldehyde in PBS for 12 h at 4°C, immersed in 30% sucrose until embedded in Cryomount (Histolab, Sweden), cryosectioned at 10 μm, and stored in -70°C. Upon use, slides were hydrated according to standard procedures and sections were incubated with background sniper (Histolab, Sweden) for 5 min. Slides were rinsed and incubated over night at 4°C with rabbit polyclonal occludin antibody 1:50 (Invitrogen) or rabbit polyclonal ZO-1 antibody 1:100 (Life Technologies). Slides were rinsed and incubated with 1:200 Alexa Fluor 546-conjugated goat-anti-rabbit antibody (Invitrogen) for 1h at room temperature (RT). Slides were rinsed and mounted with Prolong® Gold-DAPI (Life Technologies). One transversal section/individual was used for each staining, and negative controls were included in all experiments. Images were acquired with a LSM700 Zeiss Inverted Confocal with a ×100 oil immersion objective. Quantification of tight junction protein expression was performed in a blinded manner by a single investigator. Z-stack images were processed in ImageJ Fiji software (NIH, Bethseda, MD) being reconstructed to Z-stack projections of the Sum intensity. The apical side of intestinal epithelial cells was manually selected by drawing a segmented line of 100 pixels width. The ROI was straightened and the average of integrated density was measured after threshold.

Giemsa Staining of Biopsies

To identify the presence of intestinal bacteria at baseline, one paraffin-embedded biopsy/individual, was stained with routine Giemsa. Coded slides were evaluated with 600× magnification using Nikon E800 microscope connected to software NIS elements (Nikon Instruments Inc. Tokyo, Japan). Bacteria in 10 areas of 0.033 mm^2/biopsy were counted and results are presented as bacteria/mm^2.

Preparation of Biopsy Lysates

Frozen biopsies were immersed in lysing buffer containing ice-cold RIPA-buffer (Pierce, Thermo Scientific, Sweden), Mini Protease inhibitor Cocktail (Roche, Germany) and 50U/mL nuclease (Pierce). Samples were homogenized in Tissue-Lyser II (Qiagen, Sweden), 30 frequency/s, for 1 min and then centrifuged for 15 min at 18,000 g at 4°C. Supernatants were redrawn, protein concentrations measured according to the Bio-Rad DC protein assay (Bio-Rad, Sweden), and lysates were diluted in Laemmli sample buffer (Bio-Rad) to a final concentration of 1 mg protein/mL, heated for 9 min at 95°C, and kept at -80°C until analysis.

Quantification of VIP in Plasma and Biopsies by EIA

Undiluted plasma and biopsy lysates, positive and negative controls, and standard points were added in duplicates. Absorbance was measured at 450 nm in VERSAmax Tunable Microplate Reader (Molecular Devices, CA) using Softmax pro 5 (Molecular Devices). The software generated a standard curve based on the standard points, from which the concentrations of the samples were calculated.

Quantification of MC-Tryptase in Biopsies by Western Blot

Biopsy lysates with protein concentrations of 20μg/μL were loaded on 8%-16% Criterion TGX gels (Bio-Rad), separated and electrophoretically transferred to nitrocellulosa membranes (Bio-Rad). Membranes were blocked for 1 h in 5% non–fat milk (Bio-Rad) in PBS pH 7.6+0.05% Tween20 at RT. Membranes were incubated at 4°C over night with mouse monoclonal-anti-human MC tryptase antibody 1:200 (Santa Cruz Biotechnology, Germany) and rabbit monoclonal-anti-human β-actin (Cell Signaling, Bio-Nordika, Sweden), 1:1000 in 5% BSA. After washing, membranes were incubated with 1:5000 secondary antibodies goat polyclonal-anti-mouse Alexa Fluor 790 or goat polyclonal-anti-rabbit Alexa Fluor 680 (Invitrogen, Sweden) for 1 h at RT. Membranes were washed and fluorescent bands were detected and quantified by Odyssey CLx and Image Studio software (LI-COR Biosciences).

Quantification of MC-Tryptase, VIP and VPACs

Biopsies were stained following the same protocol described for tight junctions above. Primary antibodies used were mouse monoclonal-anti-human MC-tryptase 1:200 (Santa Cruz)+goat polyclonal-anti-human VPAC1/VPAC2 1:400 (Santa Cruz) or goat polyclonal VIP 1:25 (Santa Cruz), and secondary antibodies 1:400 Alexa Fluor 594-conjugated rabbit-anti-mouse (Life Technologies, Sweden) and 1:200 Alexa Fluor 488-conjugated donkey-anti-goat (Life technologies). Sections were evaluated in a blinded fashion by two independent researchers in a Nikon E800 fluorescence microscope connected to software NIS elements. Three sections/individual were stained for each double-staining, and negative controls were included in all experiments. The total number of MCs, and co-localization with either VPACs or VIP were quantified manually at 400× magnification. Approximately 8 area-units/section were counted and every area-unit had the same size and only area-units that were covered completely by tissue were used. The total number of MCs was quantified, followed by the number of cells positive for both tryptase and VIP/VPAC1/VPAC2, respectively. The percentage of MCs expressing VIP/VPAC1/VPAC2 was calculated and the mean value for each section was estimated for MC-tryptase expression only as well as the percentage of MCs expressing VIP/VPAC1/VPAC2. Finally, a median value was calculated from all means to create a median value of the number of MCs and percentages of VIP/VPAC1/VPAC2 positive MCs from patients with IBS and HCs, respectively.

Supplementary Table 1. Effects on Tight Junctions by *Salmonella typhimurium*

Occludin (% of vehicle)	*S typhimurium*	*S typhimurium*+ Anti-VPACs	*S typhimurium*+ ketotifen
IBS	**42.9** [21.8-80.1]	**61.6** [29.0-88.4]	**130.5** [100.2-154.6][a,b]
HC	**47.3** [10.7-105.8]	**97.3** [56.7-178.0]	**183.2** [107.5-284.3]
ZO-1 (% of vehicle)	***S typhimurium***	***S typhimurium*+ Anti-VPACs**	***S typhimurium*+ ketotifen**
IBS	**64.7** [40.7-107.3]	**83.0** [77.9-98.5]	**122.1** [68.6-163.0]
HC	**86.4** [77.3-94.5]	**77.6** [67.1-99.2]	**61.8** [53.4-98.5]

NOTE. Colonic biopsies from 4 patients with irritable bowel syndrome (IBS) and 4 healthy controls (HC) were mounted in Ussing chambers and added buffer (vehicle), *S typhimurium*, *S typhimurium*+vasoactive intestinal polypeptide receptor blocker (anti-VPACs) or *S typhimurium*+ketotifen. Biopsies were stained for occludin and ZO-1 by immunofluorescence. Expressions were evaluated by confocal microscopy and results were normalized and presented as % of the expression in vehicle biopsies set to 100%.

[a] $P < .05$ vs vehicle. Comparisons were done with Mann-Whitney U test and values given as (median [25th-75th percentile]).

[b] $P < .005$ vs *S typhimurium*. Comparisons were done with Mann-Whitney U test and values given as (median [25th-75th percentile]).